VIROLOGY 238, 372–379 (1997)
ARTICLE NO. VY978855

Negative Regulation of a Heterologous Promoter by Human Cytomegalovirus Immediate-Early Protein IE2

Hsiu-Lan Tsai,*,† Guang-Hsiung Kou,† Fen-Mei Tang,* Cheng-Wen Wu,* and Young-Sun Lin*,[1]

*Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan; and †Department of Zoology, National Taiwan University, Taipei 10764, Taiwan

Received June 16, 1997; returned to author for revision August 27, 1997; accepted September 17, 1997

The HCMV IE2 protein promiscuously activates transcription of many viral and cellular genes. IE2 also negatively autoregulates its own expression by binding to a strategically positioned IE2 binding site, called CRS, located immediately downstream of the TATA box of the HCMV major IE promoter. Here we show that IE2 is able to repress transcription driven by a heterologous promoter, RSV LTR. Repression of RSV LTR by IE2 is completely dependent of DNA sequences downstream of the TATA box of RSV LTR. A DNA sequence, 5′-CGATACAATAAACG-3′, evidently matching the proposed CRS consensus sequence, is located between nucleotides −13 and +1 (relative to the transcription start site) of RSV LTR. Three lines of evidence support the notion that this RSV CRS element is involved in the IE2-mediated repression of RSV LTR. First, introduction of mutation to the RSV CRS element renders to the mutant RSV LTR resistance to IE2-mediated repression. Second, a mutant IE2 defective in DNA binding cannot downregulate transcription from RSV LTR. Third, IE2 specifically binds to the wild-type, but not the mutant, RSV CRS element *in vitro.* These data, in conjunction with previous works, demonstrate that IE2 can passively repress transcription of homologous and heterologous promoters that contain a CRS element.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the beta subgroup of herpes viruses, has a double-stranded DNA genome of 229,354 base pairs with a potential to encode for more than 200 proteins (Chee *et al.,* 1990). HCMV infection is detected in 40 to 100% of the population (Macias *et al.,* 1996). Infection of a healthy adult is usually asymptomatic and the HCMV genome remains in a latent state in monocytes or granulocyte–macrophage precursor cells (Taylor-Wiedeman *et al.,* 1991; Kondo *et al.,* 1994). In contrast, infection of the unborn and immunocompromised can lead to devastating consequences (Alford and Britt, 1990).

In tissue culture studies, HCMV is characterized by its narrow host range and prolonged replicative cycle. A number of immediate-early (IE) proteins of HCMV are made immediately following entry of the virus into cells (Wathen and Stinski, 1982). Among these proteins, the IE2 86K protein (referred to hereafter as IE2) is the most studied. IE2 is able to promiscuously transactivate viral and cellular gene expression (Pizzorno *et al.,* 1988; Malone *et al.,* 1990; Hagemeier *et al.,* 1992) as well as to negatively autoregulate its own promoter, the HCMV major IE promoter (MIEP) (Pizzorno and Hayward, 1990; Lang and Stamminger, 1993). Transactivation by IE2 involves interactions of the viral protein with both general transcription factors (Caswell *et al.,* 1993) and gene-specific factors (Scully *et al.,* 1995; Lang *et al.,* 1995). The interaction with the general transcription factor TBP (Caswell *et al.,* 1993) may provide an explanation for the promiscuity of transactivation by IE2.

In contrast, the ability of IE2 to negatively autoregulate is attributed to its sequence-specific DNA-binding activity (Pizzorno and Hayward, 1990; Lang and Stamminger, 1993). A consensus IE2-binding site typically contains two copies of the dinucleotide CG, separated by 10 not well-conserved, but A/T-rich, nucleotides (Arlt *et al.,* 1994). MIEP contains an IE2-binding site, strategically positioned immediately downstream of its TATA box and is called the "*cis* repression signal" (CRS) for its role in the negative autoregulation (Pizzorno and Hayward, 1990; Lang and Stamminger, 1993). Presumably, blocking assembly of the transcription initiation complex on the promoter by steric hindrance caused by binding of IE2 to CRS possibly underlines the mechanism for IE2-mediated repression of MIEP (Lee *et al.,* 1996). Thus, transcriptional repression by IE2 via the CRS pathway is both passive and position-dependent. To date, MIEP is the only promoter known to be passively downregulated by IE2. As might be expected, a heterologous promoter is negatively regulated by IE2 as well, once an IE2-binding site is, as in the case of MIEP, strategically positioned on the promoter. (For simplicity, we will refer to this particular type of IE2-binding site as CRS hereafter.)

In this report we demonstrate that IE2 represses RSV

[1] To whom correspondence should be addressed. Fax: 886-2-7853569. E-mail: bmyslin@ccvax.sinica.edu.tw.

0042-6822/97 $25.00

LTR. The IE2-mediated transcriptional downregulation of RSV LTR has all the characteristics of autoregulation of MIEP by IE2. The current work, in conjunction with previous studies, indicates that IE2 can function as a passive transcriptional repressor for both homologous and heterologous promoters.

MATERIALS AND METHODS

Plasmid construction

Plasmids pUTKAT1, pCATwt760, pRSVCAT, pIE2, and pGEM4IE2 have been previously described (Gorman *et al.,* 1982; Prost and Moore, 1986; Pizzorno *et al.,* 1991; Tsai *et al.,* 1996). pIE2(H446L,H452L), which is otherwise identical to pIE2 except that histidine at residues 446 and 452 of IE2 is substituted by leucine, was constructed by PCR-mediated mutagenesis (Barik and Galinski, 1991). pRSV(−229/−21)CAT was constructed by replacing the *Hin*dIII/*Bam*HI fragment of pE1bCAT with a PCR-generated DNA fragment containing nucleotides −229 to −21 of RSV LTR. pRSV(−229/+34)CAT was constructed by replacing the *Sph*I/*Bam*HI fragment of pRSV(−229/−21)CAT with the *Sph*I/*Hin*dIII fragment of pRSVCAT. pRSV(−538/−21)CAT was constructed by replacing the *Xho*I/*Sph*I fragment of pRSV(−229/−21)CAT with the *Nde*I/*Sph*I fragment of pRSVCAT. pRSV(−538/+34)pm5CAT was constructed by mutating nucleotides −13 to −9 of RSV LTR from 5′-CGATA-3′ to 5′-ATGGC-3′. p8HisIE2C was constructed by inserting a DNA fragment encoding IE2 residues 290–579 between the *Nde*I and *Bam*HI sites of 8His-pET11d (Hoffmann and Roeder, 1991).

Cell culture, transfection, and CAT assay

The lung cancer cell line H1299 (ATCC CRL-5803) (Unger *et al.,* 1992; Tsai *et al.,* 1996) and HeLa cells were maintained in DMEM with 10% fetal calf serum. Approximately 6×10^5 cells were seeded 12 h before transfection. Calcium phosphate-mediated DNA transfection was performed as previously described (Tsai *et al.,* 1996). Five micrograms of a LacZ reporter plasmid pCH110 (Pharmacia) was included to monitor the transfection efficiency. Typically, the transfection lasted 12 h. CAT activity was measured 48 h following transfection and quantitated as previously described (Hsu *et al.,* 1995; Tsai *et al.,* 1996).

In vitro translation of IE2 protein

Plasmid pGEM4IE2 was used to program rabbit reticulocyte lysate for the synthesis of IE2. *In vitro* transcription/translation was performed with the TNT system (Promega) according to the manufacturer's instructions.

DNA-binding assay

^{32}P-end-labeled probes for DNA-binding studies were the RSV-LTR CRS oligonucleotide 5′-AGCTCGATACAATAAACGCCGGCC-3′ and the mutant RSV-LTR CRS oligonucleotide 5′-AGCTATGGCCAATAAACGCCGGCC-3′. DNA-binding assays were performed as previously described (Kern *et al.,* 1991; Hsu *et al.,* 1995), except that *in vitro* translated IE2 and an anti-IE2 monoclonal antibody (Du Pont) were used. In brief, approximately 0.1 pmol of each probe and 100 ng of (poly dG–dC)$_2$ were incubated with an equal amount (5 μl) of *in vitro* translated IE2 protein in 100 μl of reaction buffer (100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40, and 5 mM DTT) at 4°C for 2 h. Then, 0.4 μg of an anti-IE2 monoclonal antibody (Du Pont) was added and the incubation continued for another 1 h at 4°C. The DNA–protein complex was precipitated by centrifugation after the addition of 25 μl of protein A–Sepharose beads (Sigma) and 10 μg of (poly dG–dC)$_2$. The collected beads were washed with 1 ml of the reaction buffer three times and the bound DNA was analyzed by electrophoresis on a 20% denaturing polyacrylamide gel.

Western immunoblotting

An equal amount (approximately 50 μg) of proteins from extracts of transfected H1299 cells was boiled in a sample buffer (125 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 20% glycerol, 0.005% bromophenol blue) for 5 min and then loaded onto a 10% SDS–polyacrylamide gel. Following electrophoresis, proteins were transferred to an Immobilon membrane (Millipore). IE2 derivatives were detected with an antibody against IE2 (Du Pont), using the ECL system (Amersham) according to the manufacturer's instructions.

Purification of recombinant 8HisIE2(290–579) and EMSA

Recombinant 8HisIE2(290–579) was expressed in and purified from *Escherichia coli* strain JM109DE3 transformed with plasmid p8HisIE2C. The his-tagged protein was purified by nickel chelating resins (Qiagen) according to the manufacturer's suggestions. For electrophoretic mobility shift assay (EMSA), 0.01 pmol of ^{32}P-end-labeled RSV-LTR CRS oligonucleotide (5′-AGCTCGATACAATAAACGCCGGCC-3′), 0.1 μg of 8HisIE2(290–579), 1 μg of (poly dI–dC)$_2$, and an indicated amount of the competitor DNA were incubated in 40 μl of a buffer containing 12.5 mM Hepes (pH 8.0), 12.5% glycerol, 60 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EDTA at room temperature for 15 min. Samples were subjected to electrophoresis at 4°C in a 5% nondenaturing polyacrylamide gel containing 0.5× TBE.

RESULTS AND DISCUSSION

Transcriptional repression of a heterologous promoter by IE2

Many nonpermissive cell lines, for example, H1299, HeLa, Saos-2, CV-1, and Vero (Pizzorno *et al.,* 1988; Liu

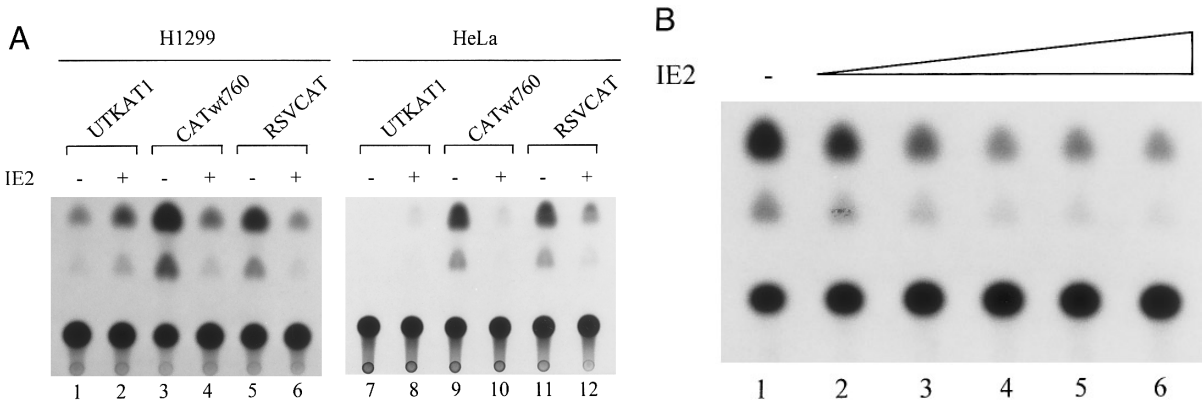

FIG. 1. HCMV IE2 differentially modulates gene transcription. (A) Repression of RSV LTR by IE2 in H1299 cells (lanes 1–6) or HeLa cells (lanes 7–12). One microgram each of the reporters and 5 μg of either the vector or the IE2 expression plasmid were transfected per sample. Experiments were repeated three times. An autoradiogram of a typical experiment is shown. The promoter element to drive the expression of assayed reporters is as follows: HSV thymidine kinase promoter for UTKAT1, HCMV major IE promoter/enhancer for CATwt760, and RSV LTR for RSVCAT. The reporter and the presence (+) or absence (−) of IE2 are indicated above the autoradiogram. The relative CAT activity (or RCA) of IE2-repressible reporters (i.e., CATwt760 and RSVCAT) was set at 100 in the absence of IE2, while the RCA of reporter UTKAT1 was set at 1. The RCA (±standard deviation) were 1 (±0), 2.4 (±0.6), 100 (±0), 4.5 (±0.3), 100 (±0), 9.7 (±1.3), 1 (±0), 3.2 (±0.7), 100 (±0), 2.9 (±0.6), 100 (±0), and 25.5 (±5.6) for lanes 1 to 12, respectively. (B) Repression of RSV LTR over a wide range of the IE2 expression plasmid. Transfection was performed with H1299 cells as described for A, except that the assayed reporter is driven by RSV LTR and that the amounts of IE2 expression plasmid transfected were 0, 1, 2, 3, 5, and 10 μg for lanes 1–6, respectively. The RCA (±standard deviation) were 100 (±0), 43.5 (±7.8), 22.7 (±4.7), 13.5 (±2.6), 11.7 (±1.9), and 10.1 (±1.7) for lanes 1 to 6, respectively.

et al., 1991; Colberg-Poley *et al.,* 1992; Hagemeier *et al.,* 1994; Lukac *et al.,* 1994; Zhu *et al.,* 1995; Lang *et al.,* 1995; Huang and Stinski, 1995; Macias *et al.,* 1996; Schwartz *et al.,* 1996; Tsai *et al.,* 1996), as well as permissive human fibroblast cells have been used to assay the transcriptional regulatory activities of IE2. Furthermore, it has been demonstrated that IE2 negatively autoregulates its own promoter in both permissive (Stenberg *et al.,* 1990; Hermiston *et al.,* 1990; Liu *et al.,* 1991) and nonpermissive cells (Pizzorno *et al.,* 1988; Pizzorno and Hayward, 1990; Liu *et al.,* 1991; Macias *et al.,* 1996), indicating that CRS-dependent repression by IE2 is largely independent of the cellular environment. To examine whether IE2 can repress transcription driven by a heterologous promoter, the influence of IE2 on the expression of several reporter constructs currently available was tested in H1299 cells, using SV40-LacZ (that is, plasmid pCH110) as an internal control to monitor the transfection efficiency. The reason to choose pCH110 as an internal control for the study is that IE2 appears to exhibit little effect on transcription driven by the SV40 promoter/enhancer (Pizzorno and Hayward, 1990; Kothari *et al.,* 1991; Liu *et al.,* 1991; Colberg-Poley *et al.,* 1992; Tsai *et al.,* 1996; and data not shown). Figure 1A shows that reporters driven by RSV LTR were repressed by IE2 (compare lane 5 to lane 6). As a positive control, the influence of IE2 on reporter pCATwt760 which is driven by MIEP was also measured (compare lanes 3 and 4). The IE2-mediated repression of MIEP and RSV LTR was specific, because under the same conditions IE2 activated, in agreement with previous studies (Pizzorno *et al.,* 1988; Malone *et al.,* 1990; Hagemeier *et al.,* 1992), transcription of the reporter under the control of the HSV TK promoter (compare lanes 1 and 2) and many other promoters (data not shown). Repression of RSV LTR by IE2 was restricted neither to H1299 cells nor to a specific amount of the IE2 expression plasmid, because a similar conclusion was reached with experiments done in HeLa cells (Fig. 1A, lanes 7–12) and because IE2-mediated repression of RSV LTR was observed over a wide range of the transfected IE2 expression plasmid (Fig. 1B). We thus concluded that IE2 specifically repressed transcription of both homologous and heterologous promoters.

DNA sequences located immediately downstream of the TATA box are involved in the IE2-mediated repression of RSV LTR

To map the promoter region of RSV LTR required for the repression by IE2, a series of reporters driven by derivatives of RSV LTR were constructed. Figure 2 illustrates that derivatives of RSV LTR remained IE2-repressible so long as they retained the −20/+34 sequence of RSV LTR (compare lanes 1 and 7 to lanes 2 and 8). In contrast, derivatives of RSV LTR with the −20/+34 sequence deleted became resistant to IE2-mediated repression (compare lanes 3 and 5 to lanes 4 and 6). These results imply that DNA sequences located immediately downstream of the RSV TATA box, which is situated between −30 and −24 of RSV LTR, are involved in the repression. In other words, IE2-mediated repression of RSV LTR seemed reminiscent of the negative autoregula-

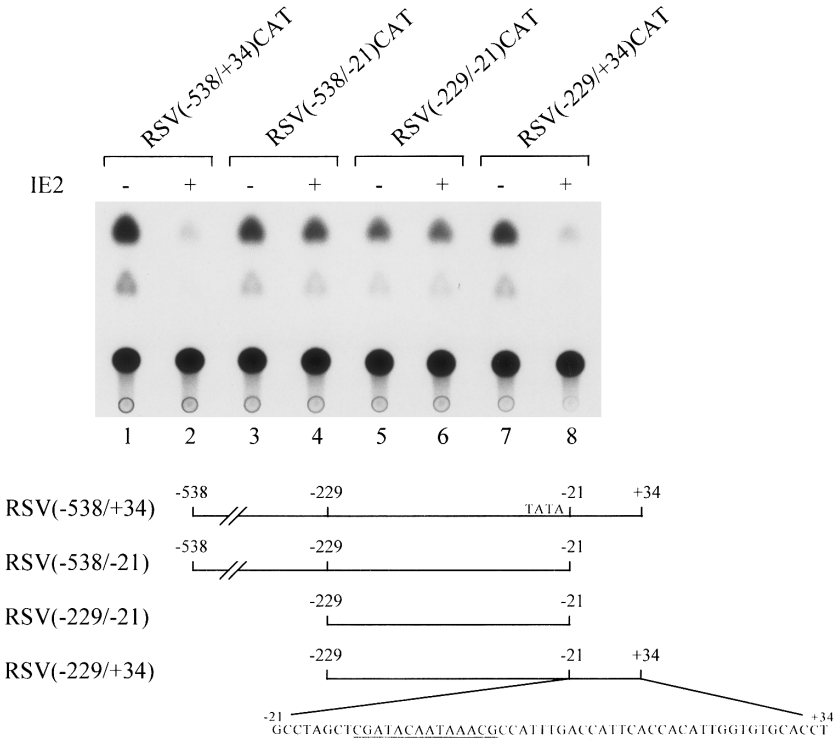

FIG. 2. IE2-mediated repression of RSV LTR requires DNA sequences downstream of the TATA box. Transfection was performed with H1299 cells as described in the legend to Fig. 1A, except that the assayed reporters are driven by derivatives of RSV LTR whose diagrams are shown below the autoradiogram, with the CRS element underlined. Otherwise as in Fig. 1A. The RCA (±standard deviation) were 100 (±0), 7.0 (±1.3), 50.4 (±10.6), 43.9 (±9.0), 23.7 (±5.6), 22.6 (±6.8), 47.7 (±10.5), and 8.7 (±1.7) for lanes 1 to 8, respectively.

tion of MIEP, suggesting the existence of a CRS element in the −20 to +34 sequences of RSV LTR. Further support for this notion is provided below.

The CRS element of RSV LTR is required for its negative regulation by IE2

In fact, a DNA sequence, 5′-CGATACAATAAACG-3′, evidently matching the proposed CRS consensus sequence (Arlt *et al.,* 1994), is located within the −20/+34 sequence of RSV LTR (Fig. 2). This RSV CRS element shares all the characteristics of IE2 binding sites: it contains two copies of the dinucleotide CG, separated by 10 not well-conserved, but A/T-rich, nucleotides (Arlt *et al.,* 1994). In terms of spatial arrangement, the location of the RSV CRS element (nucleotides −13 to +1 of RSV LTR) closely mimics that of MIEP (nucleotides −14 to −1 of MIEP) (Lang and Stamminger, 1993), strongly suggesting that the RSV CRS element is, likewise, strategically positioned so that IE2, when binding with this element, is able to block assembly of the preinitiation complex by steric hindrance. To test this idea, the RSV-LTR CRS element was mutated and the effect of IE2 on the resultant RSV LTR mutant was subsequently measured. As shown in Fig. 3, the introduction of mutation to the CRS element had little effect on the basal promoter activity of RSV LTR (compare lane 3 to lane 1). In contrast, while wild-type RSV LTR was greatly repressed by IE2 (lanes 1 and2), mutant RSV LTR was completely callous to the presence of IE2 (lanes 3 and 4). Moreover, the observation that the mutant RSV LTR was not repressed by IE2 ruled out the trivial possibility that IE2 repressed RSV LTR via a nonspecific mechanism. We thus concluded that the CRS element was indispensable for IE2-mediated repression of RSV LTR.

The DNA-binding activity of IE2 is absolutely required for the repression of RSV LTR

Data shown in Fig. 3 suggest that binding of IE2 to the CRS element resulted in the inhibition of RSV LTR. To provide further evidence for this notion, the requirement of IE2′s DNA-binding activity for repression of RSV LTR was then determined. As shown in Fig. 4, wild-type IE2 downregulated both MIEP and RSV LTR (left panel, compare lanes 2 and 5 to lanes 1 and 4). In contrast, IE2(H446L, H452L), a mutant IE2 devoid of DNA-binding activity (Huang and Stinski, 1995), repressed neither MIEP nor RSV LTR (left panel, compare lanes 3 and 6 to lanes 1 and 4). The failure of IE2(H446L, H452L) in repressing the two promoters was not due to a difference in the level of protein synthesis, since both wild-type and mutant IE2 proteins were made to similar levels (right panel, compare lane 2 to lane 3). On the basis of these experiments, it was concluded that DNA-binding activity

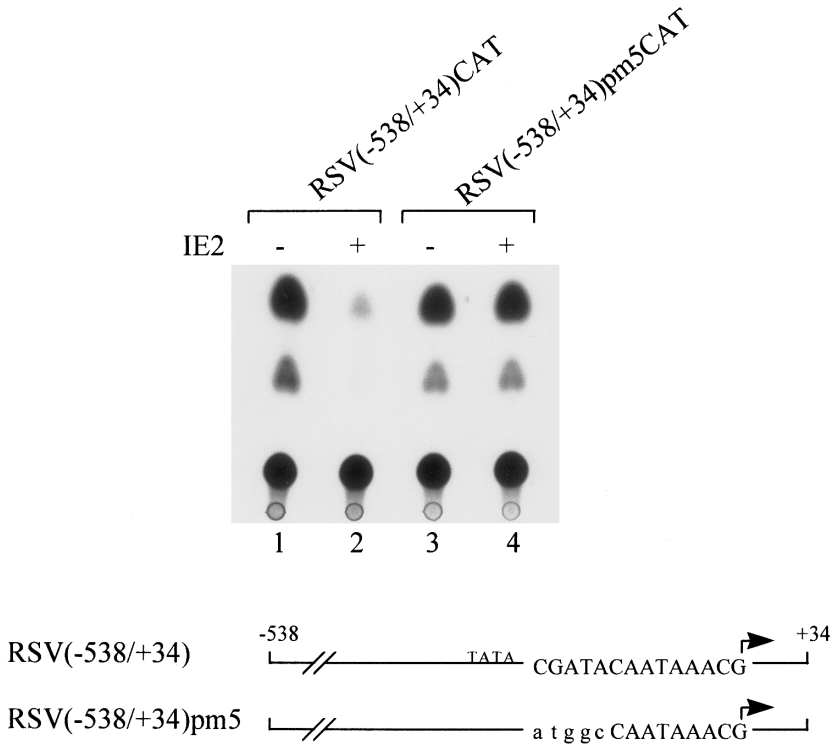

FIG. 3. Relief of IE2-mediated repression of RSV LTR by mutating its CRS element. Transfection was performed with H1299 cells as described in the legend to Fig. 1A, except that the assayed reporters are driven by derivatives of RSV LTR whose diagrams are shown below the autoradiogram, with nucleotide sequences of the wild-type and mutant CRS element displayed, respectively. Otherwise as in Fig. 1A. The RCA (±standard deviation) were 100 (±0), 11.8 (±2.1), 75.1 (±13.5), and 73.1 (±13.9) for lanes 1 to 4, respectively.

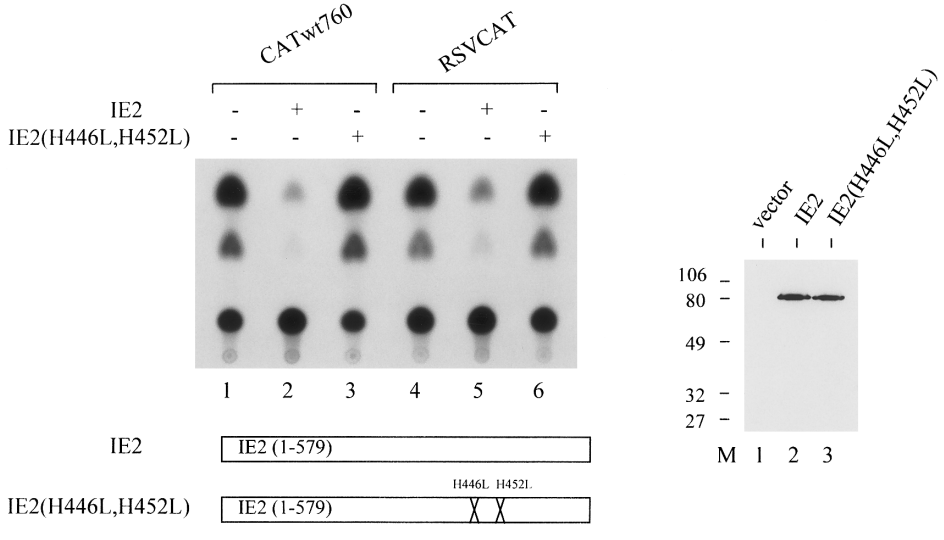

FIG. 4. IE2(H446L, H452L) fails to repress both MIEP and RSV LTR. (Left) Repression of MIEP and RSV LTR by IE2 derivatives. Transfection was performed with H1299 cells as described in the legend to Fig. 1A, except that the assayed reporters were CATwt760 and RSVCAT. The presence (+) or absence (−) of the IE2 derivative is indicated above the autoradiogram. Diagrams of the structure of IE2 derivatives are shown below the autoradiogram. Otherwise as in Fig. 1A. The RCA (±standard deviation) were 100 (±0), 7.8 (±1.4), 124.8 (±23.8), 100 (±0), 16.5 (±2.6), and 118.4 (±17.8) for lanes 1 to 6, respectively. (Right) The expression levels of IE2 derivatives. Proteins of the nuclear fraction of H1299 cells transiently transfected with the vector (lane 1) or IE2 derivatives (lanes 2 and 3) were fractionated on a 10% SDS–PAGE gel. IE2 derivatives were detected by immunoblotting as described under Materials and Methods. The IE2 derivative is indicated above each track of the immunoblot. The positions of molecular mass markers in kilodaltons are indicated on the left.

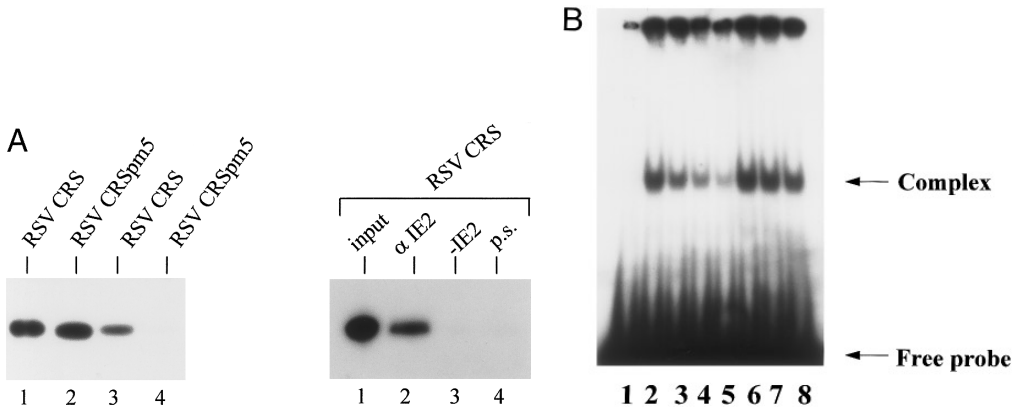

FIG. 5. IE2 specifically binds the CRS element of RSV LTR *in vitro.* (A) Immunoprecipitation of DNA–protein complex. (Left) Precipitation of the wild-type but not mutant CRS element by IE2 protein. Lanes 1 and 2, one-hundredth each of the input DNA probes directly loaded onto the gel. Lanes 3 and 4, recovery of probes by immunoprecipitation of the IE2–DNA complex. The DNA probe is indicated above each track of the autoradiogram. (Right) Precipitation of the CRS element requires both IE2 protein and anti-IE2 antibody. Lane 1, one-hundredth of the input DNA probe directly loaded onto the gel. Lane 2, recovery of the CRS probe in the presence of IE2 and an anti-IE2 antibody. Lanes 3 and 4, as in lane 2, except that IE2 was omitted (lane 3) or the anti-IE2 antibody was replaced with a preimmune serum (lane 4). (B) EMSA and cold competition assay with wild-type and mutant RSV-LTR CRS oligonucleotides. Lanes: 1, free probe; 2, probe plus recombinant IE2; 3, 4, and 5, probe and recombinant IE2 plus 10-, 20-, and 50-fold molar excesses of the wild-type RSV-LTR CRS oligonucleotide, respectively; 6, 7, and 8, probe and recombinant IE2 plus 10-, 20-, and 50-fold molar excesses of the mutant RSV-LTR CRS oligonucleotide, respectively. The IE2–DNA complex and free probe are indicated by arrows.

of IE2 was absolutely required for the negative regulation of MIEP and RSV LTR.

IE2 binds specifically to the CRS element of RSV LTR *in vitro*

Our results strongly suggest that binding of IE2 to the CRS element of RSV LTR underlies the mechanism for IE2-mediated repression of RSV LTR. A corollary of this argument was that the CRS element of RSV LTR behaved as an authentic IE2-binding site. To test this idea, the following experiments were performed. First, an immunoprecipitation study of DNA–protein interaction using a monoclonal anti-IE2 antibody was performed. As shown in Fig. 5A, IE2 bound reproducibly to the wild-type CRS element of RSV LTR, but not to the mutant one (left panel, compare lane 3 to lane 4). In other words, the mutant CRS which failed to mediate transrepression of RSV LTR by IE2 *in vivo* (Fig. 3) exhibited, as expected, little affinity toward IE2 *in vitro.* Furthermore, precipitation of the CRS DNA probe required the presence of both IE2 protein and the anti-IE2 antibody, since immunoprecipitation performed either with rabbit reticulocyte lysate that was not programmed with the IE2 expression plasmid or with the substitution of anti-IE2 antibody with a preimmune serum failed to precipitate (right panel, compare lanes 3 and 4 to lane 2), indicating that binding of the CRS element by IE2 was prerequisite for its recovery in the immunoprecipitation. Nonetheless, since the above data were collected with *in vitro* translated IE2 protein, it was possible that some reticulocyte lysate's protein(s) was also required for the binding. To rule out this possibility, the IE2–DNA binding domain was expressed in and purified from *E. coli* and used in an electrophoretic mobility shift assay. Figure 5B shows that recombinant IE2 formed a complex with the RSV-LTR CRS oligonucleotide (lane 2). Furthermore, the complex was much more sensitive to competition by the wild-type RSV-LTR CRS oligonucleotide than by the mutant one (compare lanes 3–5 to lanes 6–8). We thus concluded that IE2 bound specifically and directly to the RSV-LTR CRS element.

On the basis of both genetic and biochemical studies, we conclude that IE2-mediated repression of a heterologous promoter, RSV LTR, shares all the characteristics of negative autoregulation of MIEP by IE2 (Pizzorno *et al.,* 1988; Stenberg *et al.,* 1990; Hermiston *et al.,* 1990; Pizzorno and Hayward, 1990; Liu *et al.,* 1991; Macias *et al.,* 1996): (1) the requirement of an IE2-binding site strategically positioned immediately downstream of the TATA box (Figs. 2, 3, and 5) and (2) the indispensability of DNA-binding activity of IE2 (Fig. 4). It is worthwhile to note that our study was mainly performed in the p53-null H1299 cell line. This observation, in conjunction with previous works with human foreskin fibroblast (HFF) cells (Hermiston *et al.,* 1990; Liu *et al.,* 1991), which contain wild-type p53, indicates that the CRS-dependent repression by IE2 appears independent of the endogenous p53 status. Recently, it has been shown that IE2 does not impede promoter recognition by TFIID, yet very effectively blocks recruitment of RNA polymerase II (Lee *et al.,* 1996). Since the spatial arrangement of the CRS element on RSV LTR closely mimics that of MIEP, it is speculated that this mechanism of repression might also be used in the control of RSV LTR by IE2. Nonetheless, it must be pointed out that RSV LTR has been shown to be marginally repressed by IE2 in HEF cells (Liu *et al.,* 1991). Cur-

TABLE 1

Derivation of a Consensus IE2-Binding Sequence

Promoter	Sequences	Position
MIEP	a a g c a g a g c t C G T T T A G T G A A C C G t c a g a t c g c c	−24 to + 10
2.2-kb RNA promoter	a g t a g c g t t g C G A T T T G C A G T C C G c t c c g g t g t c	−153 to −120
	t c t c a g g a g g C G G A A A G G A A A T C G g a t a a c g g c a	−253 to −240
	t g t t a g g t t g C G G A G A T A A G T C C G t g a t t a g t c g	−291 to −258
1.2-kb RNA promoter	g a c t g g c g t g C G A C C T G T A A A C C G t t a c t c g g g t	−130 to −97
RSV LTR	g t g c c t a g c t C G A T A C A A T A A A C G c c a t t t g a c c	−23 to +11
Consensus IE2-binding site	C G N N N N N N N N N N C G	
	A/T-rich	

Note. Sequences of six IE2-binding sites identified in this and previous (Arlt *et al.,* 1994; Schwartz *et al.,* 1994; Scully *et al.,* 1995) studies. The sequences are aligned by the two copies of the invariant dinucleotide CG within the consensus IE2-binding site. One additional IE2 site was identified in a region upstream of the HCMV UL4 promoter (Huang and Stinski, 1995). This site, however, does not fit the proposed consensus IE2-binding sequence and is therefore omitted.

rently, we do not know the reason for the observed discrepancy.

The six IE2-binding sites defined in this and previous (Arlt *et al.,* 1994; Schwartz *et al.,* 1994; Scully *et al.,* 1995) studies are aligned to derive a consensus IE2-binding site (Table 1). Based on this consensus sequence, we have identified seven human genes with an IE2-binding site situated either in the immediate downstream of or overlapping with the TATA box (data not shown). Since the spatial arrangement of the newly identified cellular IE2-binding sites is slightly different from that of MIEP and RSV LTR, additional experiments are required, however, to address if these cellular genes can be downregulated by IE2 via the CRS mechanism.

ACKNOWLEDGMENTS

We acknowledge Dr. G. S. Hayward for plasmid pCATwt760. This work was supported by grants from the Academia Sinica and the National Science Council of Taiwan to G.H.K., C.W.W., and Y.S.L.

REFERENCES

Alford, C. A., and Britt, W. J. (1990). Virology. Raven Press, New York.

Arlt, H., Lang, D., Gebert, S., and Stamminger, T. (1994). Identification of binding sites for the 86-kilodalton IE2 protein of human cytomegalovirus within an IE2-responsive viral early promoter. *J. Virol.* 68, 4117–4125.

Barik, S., and Galinski, M. S. (1991). "Megaprimer" method of PCR: Increased template concentration improves yield. *Biotechniques* 10, 489–490.

Caswell, R., Hagemeier, C., Chiou, C. T., Hayward, G. S., Kouzarides, T., and Sinclair, J. H. (1993). The human cytomegalovirus 86K immediate early (IE) 2 protein requires the basic region of the TATA-box binding protein (TBP) for binding and interacts with TBP and transcription factor TFIIB via regions of IE2 required for transcriptional regulation. *J. Gen. Virol.* 74, 2691–2698.

Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., Kouzarides, T., and Martignetti, J. A. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Topics Microbiol. Immunol.* 154, 125–169.

Colberg-Poley, A. M., Santomenna, L. D., Harlow, P. P., Benfield, P. A., and Tenney, D. J. (1992). Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. *J. Virol.* 66, 95–105.

Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA* 79, 6777–6781.

Hagemeier, C., Walker, S. M., Sissons, P. J., and Sinclair, J. H. (1992). The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently trans-activate the c-fos, c-myc and hsp70 promoters via basal promoter elements. *J. Gen. Virol.* 73, 2385–2393.

Hagemeier, C., Caswell, R., Hayhurst, G., Sinclair, J., and Kouzarides, T. (1994). Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. *EMBO J.* 13, 2897–2903.

Hermiston, T. W., Malone, C. L., and Stinski, M. F. (1990). Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. *J. Virol.* 64, 3532–3536.

Hoffmann, A., and Roeder, R. G. (1991). Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. *Nucleic Acids Res.* 19, 6337–6338.

Hsu, Y. S., Tang, F. M., Liu, W. L., Chuang, J. Y., Lai, M. Y., and Lin, Y. S. (1995). Transcriptional regulation by p53: Functional interactions among multiple regulatory domains. *J. Biol. Chem.* 270, 6966–6974.

Huang, L., and Stinski, M. F. (1995). Binding of cellular repressor protein or the IE2 protein to a cis-acting negative regulatory element upstream of a cytomegalovirus early promoter. *J. Virol.* 69, 7612–7621.

Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. *Science* 252, 1708–1711.

Kondo, K., Kaneshima, K. H., and Mocarski, E. S. (1994). Human cytomegalovirus latent infections of granulocyte–macrophage progenitors. *Proc. Natl. Acad. Sci. USA* 91, 11879–11883.

Kothari, S., Baillie, J., Sissons, J. G., and Sinclair, J. H. (1991). The 21bp repeat element of the human cytomegalovirus major immediate early enhancer is a negative regulator of gene expression in undifferentiated cells. *Nucleic Acids Res.* 19, 1767–1771.

Lang, D., Gebert, S., Arlt, H., and Stamminger, T. (1995). Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. *J. Virol.* 69, 6030–6037.

Lang, D., and Stamminger, T. (1993). The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE-1/2 enhancer-promoter. *J. Virol.* 67, 323–331.

Lee, G., Wu, J., Luu, P., Ghazal, P., and Flores, O. (1996). Inhibition of the association of RNA polymerase II with the preinitiation complex by a viral transcriptional repressor. *Proc. Natl. Acad. Sci. USA* 93, 2570–2575.

Liu, B., Hermiston, T. W., and Stinski, M. F. (1991). A cis-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. *J. Virol.* 65, 897–903.

Lukac, D. M., Manuppello, J. R., and Alwine, J. C. (1994). Transcriptional activation by the human cytomegalovirus immediate-early proteins: Requirements for simple promoter structures and interactions with multiple components of the transcription complex. *J. Virol.* 68, 5184–5193.

Macias, M. P., Huang, L., Lashmit, P. E., and Stinski, M. F. (1996). Cellular or viral protein binding to a cytomegalovirus promoter transcription initiation site: Effects on transcription. *J. Virol.* 70, 3628–3635.

Malone, C. L., Vesole, D. H., and Stinski, M. F. (1990). Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: Mutational analysis of the viral proteins. *J. Virol.* 64, 1498–1506.

Pizzorno, M. C., O'Hare, P., Sha, L., Lafemina, R. L., and Hayward, G. S. (1988). Trans-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. *J. Virol.* 62, 1167–1179.

Pizzorno, M. C., Mullen, M. A., Chang, Y. N., and Hayward, G. S. (1991). The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. *J. Virol.* 65, 3839–3852.

Pizzorno, M. C., and Hayward, G. S. (1990). The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. *J. Virol.* 64, 6154–6165.

Prost, E., and Moore, D. D. (1986). CAT vectors for analysis of eukaryotic promoters and enhancers. *Gene* 45, 107–111.

Schwartz, R., Sommer, M. H., Scully, A., and Spector, D. H. (1994). Site-specific binding of the human cytomegalovirus IE2 86-kilodalton protein to an early gene promoter. *J. Virol.* 68, 5613–5622.

Schwartz, R., Helmich, B., and Spector, D. H. (1996). CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. *J. Virol.* 70, 6955–6966.

Scully, A. L., Sommer, M. H., Schwartz, R., and Spector, D. H. (1995). The human cytomegalovirus IE2 86-kilodalton protein interacts with an early gene promoter via site-specific DNA-binding and protein–protein associations. *J. Virol.* 69, 6533–6540.

Stenberg, R. M., Fortney, J., Barlow, S. W., Magrane, B. P., Nelson, J. A., and Ghazal, P. (1990). Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. *J. Virol.* 64, 1556–1565.

Taylor-Wiedeman, J., Sissons, J. G. P., Borysiewicz, L. K., and Sinclair, J. H. (1991). Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* 72, 2059–2064.

Tsai, H. L., Kou, G. H., Chen, S. H., Wu, C. W., and Lin, Y. S. (1996). Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53. *J. Biol. Chem.* 271, 3534–3540.

Unger, T., Nau, M. M., Segal, S., and Minna, J. D. (1992). p53: A transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.* 11, 1383–1390.

Wathen, M. W., and Stinski, M. F. (1982). Temporal patterns of human cytomegalovirus transcription: Mapping the viral RNAs synthesized at immediate early, early, and late times after infection. *J. Virol.* 41, 462–477.

Zhu, H., Shen, Y., and Shenk, T. (1995). Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J. Virol.* 69, 7960–7970.